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Simultaneous Cytometric Analysis of (Auto)antigen-Reactive T and B Cell Proliferation

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Abstract

The detection and characterization of (auto)antigen-specific lymphocytes, both B and T cells, is essential to investigate immunopathologic mechanisms. Our aim was to perform a CFSE (Carboxyfluorescein diacetate succinimidyl ester)-based cytometric analysis of peripheral blood mononuclear cells (PBMC) proliferating in response to antigenic provocation. CFSE-labeled PBMC were stimulated with a superantigen (SEB), a recall antigen (tetanus toxoid), an allergen (grass pollen) and an autoantigen (nucleosomes) and stained after cultivation with CD4-, CD8- and CD19-antibodies. Proliferated cells were identified cytometrically by the decrease of the CFSE fluorescence intensity due to cell division. Antigen-reactive, proliferated B cells were further analysed phenotypically, antigen-specific proliferated Th cells were further characterized functionally regarding their cytokine secretion pattern after polyclonal restimulation. Using this technique, antigen-specific proliferated B and Th cells were detected even at low frequencies. Analyzing the cytokine secretion pattern of allergen-reactive proliferated Th cells after polyclonal restimulation we found differences in the expression of IL-13 and IL-4 between an atopic and a healty donor. After stimulation of PBMC from TT-vaccinated donors TT-specific proliferated B cells were detected in high frequencies and showed a plasmablast-typical $CD20^{low}$ $CD27^{high}$ phenotype with only low frequencies expressing CD138 (= Syndecan-1). Proliferation of nucleosome-reactive Th cells and B cells was observed in both patients and healthy controls. We have optimized here the cytometric analysis of reactive cell proliferation based on CFSE offering various facilities of application on the further characterization of both antigen-specific B and T cells.

Introduction

In the past, immunofluorescence-based cytometry and cell sorting have found their place in the repertoire of state-of-the-art technologies, mostly serving to identify subsets of lymphocytes and systemic changes of the immune system. Although certainly of value for diagnosis and in particular for the analysis of immunopathology and to evaluate new therapeutic approaches, cytometry then still had one major limitation: except for a few

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experimental situations it was rather difficult to focus analysis on those specific lymphocytes recognizing the relevant antigens in a normal or pathological immune reaction. This drawback has been overcome in the meanwhile for both B and T lymphocytes, using the antigen to identify the cells. Whereas for B cells the native antigen itself, conjugated to fluorescent dyes, has been used to identify and isolate antigen-specific B cells (1), antigen-specific T cells can only be analyzed using the corresponding immunodominant peptides of the antigen in the context of MHC-multimeres (2). However, the tetramere-technology requires recombinant MHC molecules and defined peptides, which are still often unknown and thus cannot be easily used for a routine analysis of antigen-specific T cells.

Therefore, other methods to analyze antigen-specific T cells independent of distinct peptides and HLA-alleles have been introduced recently. Mostly such methods as ELISPOT or the cytometric cytokine secretion assay are based on the analysis of reactive cytokine production after short-term provocation of the cells with antigen *in vitro* (3, 4). Apart from the strict dependence of such methods on fully functional antigen presentation they can only detect or isolate T cells reacting with the production of particular cytokines. Another access to *in vivo* preactivated memory cells is their reactive proliferation induced by antigenic provocation *in vitro*. Still common, also in routine laboratories, is to perform *in vitro* proliferation assays on the basis of tritiated thymidine ([^{3}H]-thymidine) incorporated during DNA-synthesis of the dividing cells. This classical method has disadvantages, though. Firstly, it is clear nowadays that also B cells can react with antigen-specific differentiation and proliferation upon antigen provocation *in vitro*. However, the [^{3}H]-thymidine does not enable to distinguish between proliferation of different cell populations from the stimulated pool. Moreover, [^{3}H]-thymidine is added to the cultures for the last 6–24 hours at the end of a culture period of several days. Cells that underwent proliferation earlier cannot be detected. In addition, the sensitivity of [^{3}H]-thymidine incorporation assays is often reduced due to high levels of background since also non-proliferating cells can take up [^{3}H]-thymidine. Cytometric analysis of DNA-synthesis has been made possible by replacing [^{3}H]-thymidine by bromodeoxyuridine (BrdU) a thymidine analogue that is also incorporated in newly synthesized DNA. BrdU can be stained with fluorescent-conjugated monoclonal antibodies enabling a detection of proliferating cells on the single cell level. Nevertheless, further functional analysis of proliferated cells is not possible because of the required fixation of the cells (5).

This drawback has been overcome recently by performing cytometric analysis of proliferation with carboxyfluorescein diacetate succinimidylester (CFSE)-labeled cells. During the labeling procedure the membrane-permeable CFSE covalently binds to free amines of cytoplasmic proteins not affecting cellular function and viability. This provides a uniform fluorescent label to all cells prior to the antigenic challenge. Cells reacting with proliferation after *in vitro* provocation with antigen then lose 50% of their fluorescent label upon each cell division. This allows not only identification and enumeration of antigen-reactive cells, but also supplies information on the proliferative history of the cells. Moreover, the cells can be isolated alive for functional studies and molecular analysis. In immune responses to candida antigens, the CFSE technology compared favorable to the classical [^{3}H]-thymidine-uptake assay (6, 7).

The striking advantages of CFSE labeling are first, the possibility to combine this label with several surface as well as intracellular markers and second, the chance to ana-

lyse living cells, permitting the exclusion of dead cells using propidium iodide (PI) and the direct access on proliferated cells for further studies. Here we have performed cytometric analysis of reactive T and B cell proliferation after antigenic *in vitro* provocation of CFSE-labeled peripheral blood mononuclear cells (PBMC). We show that antigen-reactive proliferating T cells can be easily identified and further characterized with respect to their cytokine secretion pattern after polyclonal restimulation. Furthermore, we demonstrate that considerable high frequencies of B cells proliferate in response to the antigenic provocation *in vitro*. Such antigen-reactive proliferated B cells revealed a plasmablast phenotype. Finally, we applied the cytometric analysis of reactive proliferation with CFSE-labeled cells to evaluate autoantigen-specific T and B cell reactivities in patients suffering from systemic lupus erythematosus (SLE) and in healthy donors.

Materials and Methods

Labeling of peripheral blood mononuclear cells with carboxyfluorescein diacetate succinimidyl ester (CFSE)

Ammonium-heparinized venous blood samples were obtained from healthy donors, an atopic donor (defined by measurement of specific IgE) and SLE patients (diagnosis according to the revised ACR criteria). Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque™ PLUS (*Amersham Pharmacia Biotech AB, Sweden*) gradient separation. Labeling of PBMC with CFSE (*Molecular Probes, Eugene, OR*) was performed with modifications as described earlier (6). Briefly, PBMC were washed twice in phosphate buffered saline (PBS) and resuspended in PBS at a final concentration of 2×10^7/ml. The cell suspension was added to the staining solution of CFSE in PBS at a 1:1 ratio (final CFSE concentration 0.25 μmol). After incubation for 4 minutes at room temperature cells were washed twice with PBS containing 0.5% bovine serum albumine (BSA) (*Sigma, Germany*) in order to stop the staining reaction.

In vitro stimulation with antigen

Cultures were performed in RPMI 1640 (*GIBCO BRL, Grand Island, USA*) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine (*all supplements PAA, Linz, Austria)* and 10% autologous, complement inactivated serum. CFSE-labeled PBMC were cultivated in the presence of antigen for 3 to 7 days in 24-well-plates *(Corning Incorporated, Corning NY)* at a cell concentration of 2×10^6/ml at 37 °C and 5% CO_2. Following antigens and superantigens were used for stimulation: staphylococcal enterotoxin B (SEB, 1 μg/ml, *Sigma, Germany*), tetanus toxoid (TT, 5μg/ml, *Chiron Behring GmbH & Co, Marburg, Germany*), lyophilisized mixed grass pollen (10 μg/ml, *ALK-SCHERAX, Germany*), nucleosomes (20μg/ml, prepared as described earlier (8)). Additional probes remained without antigen. After cultivation cells were harvested and washed once with ice-cold PBS-BSA.

Magnetical separation and polyclonal restimulation of T helper cells

To isolate T helper (Th) cells from CFSE-labeled, antigen-stimulated PBMC cells were incubated with CD4 MultiSort MicroBeads *(Miltonyi Biotec, Cologne, Germany)* for 20 min. at 4 °C in the presence of 5 mg/ml Beriglobin® (*Chiron Behring GmbH & Co, Marburg, Germany*) to block unspecific binding to Fc-receptors. After subsequent washing and resuspension of the cells in PBS/BSA CD4 positive and negative cells were separated using VS^+-columns in a high gradient magnetical field *(Miltonyi Biotec)*. The purity of the positive fraction checked cytometrically was higher than 99%. Polyclonal restimulation of the $CD4^+$ cells was performed with 5 ng/ml PMA and 1 μg/ml ionomycin (*Sigma, Germany*). Cells were incubated at a concentration of $1–5\times10^6$/ml for 6 hours at 37 °C. For the last two hours 1 μg/ml brefeldin A *(Sigma, Germany)* was added as a secretion inhibitor.

Flow cytometric analysis of PBMC and T helper cells

The following antibodies were used for surface staining of PBMC: αCD4-PerCP, αCD8-PE, αCD19-PE, αCD19-PerCP, αCD19-APC, αCD20-PE, αCD20-PerCP, αCD138-PE (*Becton Dickinson Immunocytometry Systems, San Jose, CA*), αCD4 (clone TT1) and αCD27 (clone M-T271, both *Becton Dickinson*) conjugated to Cy5 (*Amersham, Braunschweig, Germany*) according to the manufacturer's instructions. Intracellular staining of Th cells was performed after fixation with formaldehyde (*Merck, Darmstadt, Germany*, 2% in PBS) for 15 minutes and permeabilization with saponine (0.5% in PBS, *Sigma, Germany*) using the cytokine antibodies: αIL-2-PE, αIL-4-PE, αIL-10-APC, αIL-13-PE, αTNF-α-APC and αINF-γ-Cy5 (*Becton Dickinson Immunocytometry Systems, San Jose, CA*). Cells were stained according to standard protocols and analysed on a FACSCalibur flow cytometer *(Becton Dickinson*). In nonfixed cells dead cells were excluded adding 1 μg/ml propidium iodide (*Sigma, Germany*) prior to FACS analysis. Data were collected and analyzed using Cellquest Research Softwear (*Becton Dickinson*).

Results

Cytometric analysis of proliferation in peripheral blood mononuclear cells after polyclonal stimulation

At first we analyzed whether antigenic provocation *in vitro* induces cell proliferation in a sufficient frequency to be detected cytometrically due to the loss of CFSE fluorescence. For this purpose we performed in the beginning polyclonal stimulations of PBMC with the superantigen SEB. CFSE-labeled PBMC were cultivated for five days with SEB (1 μg/ml) and as a control without SEB. Polyclonally stimulated cells showed a drastic sequential loss of CFSE fluorescence intensity as compared to non-stimulated cells, showing a homogenous, bright CFSE label. Using autologous serum rather than fetal calf serum as supplement to the culture medium frequencies of proliferated cells in unstimulated samples could be decreased to frequencies lower than 0.1%.

Simultaneous cytometric analysis of Tetanus-reactive proliferating T helper cells, cytotoxic T cells and B cells in one sample

Next we evaluated the simultaneous cytometric analysis of reactive T and B cell proliferation in one sample after stimulation with a recall antigen. Hereunto CFSE-labeled PBMC obtained from Tetanus-vaccinated, healthy donors were stimulated *in vitro* for 6 days with TT. Samples without antigen served as negative controls. Cultured cells were harvested and stained simultaneously with αCD4-, αCD8- and αCD19-antibodies conjugated to different fluorescent dyes. Figure 1 shows the cytometric analysis of such a sample. Interestingly, high frequencies of TT-reactive proliferated cells could be detected among $CD4^+$ T helper cells as well as among $CD19^+$ B cells. Only a small fraction of $CD8^+$ cytotoxic T cells could be detected.

Analysis of cytokine secretion in antigen-reactive proliferated Th cells after polyclonal restimulation

Apart from the plain detection of proliferation, we aimed also for a further functional analysis of antigen-reactive proliferated cells, in particular in evaluating cytokine secretion patterns. Therefore again PBMC obtained from TT-vaccinated donors were labeled

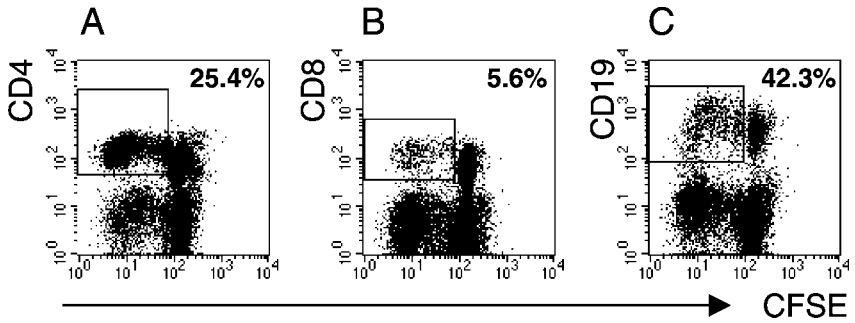

Figure 1. FACS-analysis of Tetanus-reactive $CD4^+$, $CD8^+$ and $CD19^+$ cell proliferation: CFSE-labeled PBMC were stimulated with tetanus toxoid (TT, 5 μg/ml) for six days and stained with CD4-PerCP, CD8-PE and CD19-APC antibodies. *A*: frequence of TT-reactive proliferated $CD4^+$ T helper (Th) cells. *B*: frequence of TT-reactive proliferated $CD8^+$ cytotoxic T (Tc) cells. *C*: frequence of TT-reactive proliferated $CD19^+$ B cells. Percentages refer to total numbers of $CD4^+$ Th cells, $CD8^+$ Tc cells or $CD19^+$ B cells, respectively.

with CFSE and stimulated *in vitro* for 6 days with tetanus toxoid. In order to recall the typical cytokine secretion pattern we polyclonally restimulated $CD4^+$ Th cells – which we previously separated magnetically from the TT-stimulated cultures – with PMA and ionomycin. The separation was necessary since stimulation with PMA and ionomycin causes a rapid downregulation of the CD4 molecule making cytometric determination of Th cells impossible. In the subsequent cytometric analysis of intracellulary stained INF-γ and IL-4 the CFSE label enabled the dinstinction between resting (non-proliferated/$CFSE^{high}$) and proliferated ($CFSE^{low}$) Th cells (Figure 2). Whereas TT-reactive proliferated Th cells showed high frequencies of INF-γ-producing T cells, the INF-γ production in TT-non-reactive Th cells was comparatively low. The proliferated Th helper cells furthermore showed higher amounts of IL-4 producing cells than the resting cells did.

Detection of different cytokine patterns in allergen-reactive Th cells from a healthy and an atopic donor

To evaluate whether such an experimental design is able to detect functional differences of Th cells in pathological situations, we compared cytokine patterns of allergen-reactive Th cells in a healthy and an atopic donor. Both donors showed similar frequencies of allergen-reactive proliferated $CD4^+$ Th cells. Figure 3 shows the final cytometric analysis of intracellular IFN-γ versus IL-4 and TNF-α versus IL-13 expression. Significant differences could be detected concerning the production of IFN-γ, IL-4 and IL-13 but also with respect to the frequencies of IFN-γ-IL-4 and TNF-α-IL-13 coproducing Th cells.

Antigen-reactive proliferating B cells differentiate into plasmablasts

Furthermore we aimed at characterising antigen-reactive proliferated B cells in more detail. On this, the surface markers CD27, CD20 and CD138 (= Syndecan-1) were used

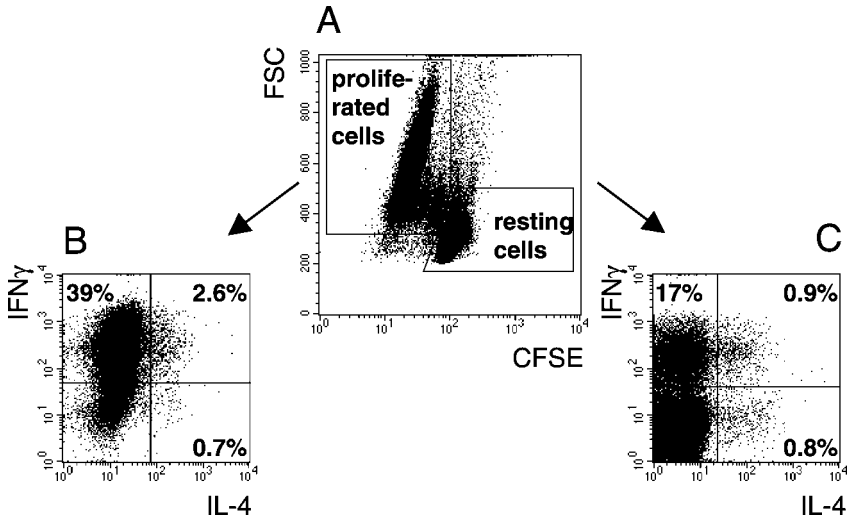

Figure 2. Cytokine expression in proliferated versus resting Th cells: CFSE-labeled PBMC were stimulated with TT (5 μg/ml) for six days. The $CD4^+$ Th cells were isolated using magnetical cells sorting (MACS) technique. After polyclonal restimulation with PMA/ionomycin intracellular staining of INF-γ and IL-4 was performed. *A:* all restimulated $CD4^+$ Th cells, electronic gating on proliferated ($CFSE^{low}$) and resting ($CFSE^{high}$) cells. *B:* INF-γ and IL-4 expression in proliferated ($CFSE^{low}$) $CD4^+$ Th cells. *C*: INF-γ and IL-4 expression in resting ($CFSE^{high}$) $CD4^+$ Th cells.

to further phenotype antigen-reactive proliferated $CD19^+$ B cells. Recently, high expression levels of surface molecule CD27 combined with the downregulation of CD20 have been shown to be characteristic for peripheral blood plasmablasts (9). Again CFSE-labeled PBMC were stimulated for six days with TT. As shown in Figure 4 electronic gating on $CD19^+$ B cells with low, medium and high CFSE fluorescense intensity allowed to assign the analysis of CD27- and CD20-expression to B cell subsets with different proliferative histories in one sample. Most of the more than 4-fold divided $CD19^+$cells showed a plasmablast phenotype in that these cells lacked the surface marker CD20 while they expressed high levels of CD27. In contrast up to 4-fold divided or resting $CD19^+$ B cells expressed CD20 and were characterised by low or medium CD27 expression levels. Thus proliferation is essential for the *in vitro* differentiation of $CD20^-$ $CD27^{++}$ $CD19^+$ plasmablasts in our experimental design.

We further phenotypically characterized the *in vitro* generated $CD27^{++}$ $CD19^+$ plasmablasts with respect to the plasma cell marker CD138. Again we stimulated CFSE labeled PBMC *in vitro* with TT and analyzed the expression of CD19, CD27 and CD138. Figure 5 shows the frequencies of $CD27^{high}$ expressing cells and of $CD27^{high}CD138^+$ cells among TT-reactive proliferated $CD19^+$ B cells at several time points (day 3, 4, 5, 6 and 7) after TT-stimulation of PBMC. Our results show that CD138, a differentiation marker of plasma cells (10), is only expressed by a small subset of TT-reactive proliferated $CD19^+$ B cells. These $CD138^+$ TT-reactive $CD19^+$ B cells can only be detected from day 6 after TT-stimulation onwards.

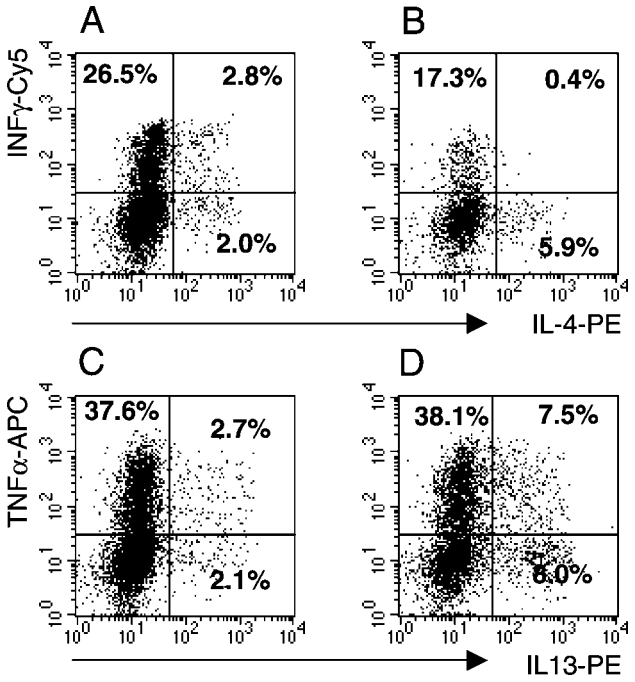

Figure 3. Differential cytokine expression of allergen-reactive $CD4^+$ Th cells in a healthy and an atopic donor: CFSE-labeled PBMC were stimulated with grass pollen (10 μg/ml) for six days. The $CD4^+$ Th cells were isolated using magnetical cells sorting (MACS) technique. After polyclonal restimulation with PMA/ionomycin intracellular staining of INF-γ , IL-4, TNF-α and IL-13 was performed. *A/C*: Cytokine expression in allergen-reactive proliferated $CD4^+$ Th cells of a healthy donor. *B/D*: Cytokine expression in proliferated allergen-reactive $CD4^+$ Th cells of an atopic donor.

Detection of autoantigen-reactive proliferated T and B cells

Finally we aimed to apply this method to the cytometric detection of autoantigen-reactive T and B cell proliferation. We obtained PBMC from 13 patients diagnosed with SLE and from 10 healthy donors, labeled them with CFSE and stimulated them with nucleosomes, one of the major targets of the humoral autoantibody response in SLE patients (11, 12). The nucleosomes were prepared from chicken erythrocytes and were also used for the evaluation of anti-nucleosomal autoantibodies in the sera of the patients. Figure 6 shows a typical analysis of nucleosome-reactive proliferation among $CD4^+$ Th cells and $CD19^+$ B cells from one of the reacting SLE patients and from one healthy donor. Nucleosome-reactive Th cell proliferation was detectable in 5 out of 13 SLE patients but also in 5 out of 10 healthy donors. Nucleosome-reactive proliferated B cells were detected in 3 patients and 2 healthy individuals. The presence of nucleosome-reactive Th or B cells in SLE-patients did not correlate with the presence of anti-nucleosomal autoantibodies and no anti-nucleosomal autoantibodies were detectable in the sera of the healthy donors (data not shown).

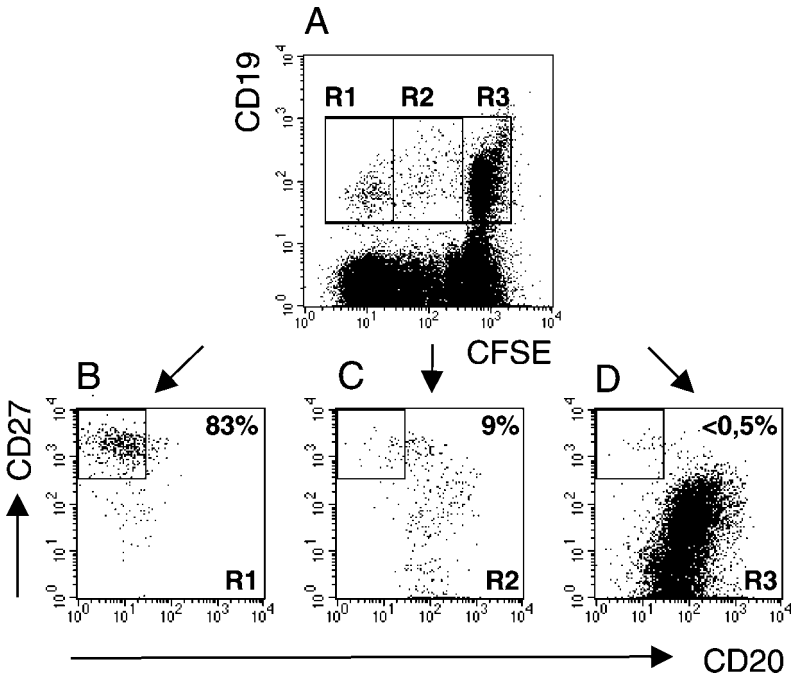

Figure 4. Expression of $CD27^{high}CD20^{low}$ phenotype in proliferated versus resting B cells: After six days of TT-Stimulation CFSE-labeled PBMC were stained with αCD19, αCD20 and αCD27 antibodies. *A*: TT stimulated CFSE-labeled PBMC, electronic gates on more than 4-fold divided ($CFSE^{low}$), up to 4-fold divided ($CFSE^{medium}$) and resting ($CFSE^{high}$) $CD19^+$ cells. *B*: CD20 and CD27 expression in more than 4-fold ($CFSE^{low}$) $CD19^+$ cells. *C*: CD20 and CD27 expression in up to 4-fold divided ($CFSE^{medium}$) $CD19^+$ cells. *D*: CD20 and CD27 expression in resting ($CFSE^{high}$) $CD19^+$ cells.

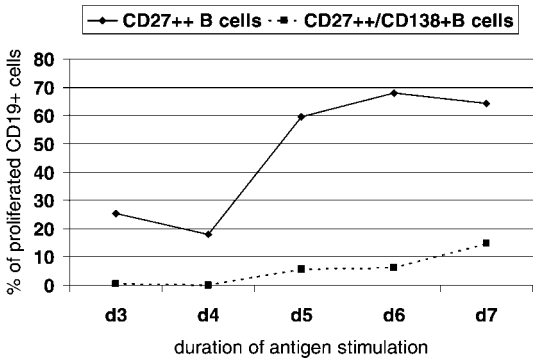

Figure 5. Expression of CD27 and CD138 during *in vitro* B cell differentiation: CFSE-labeled, TT-stimulated PBMC were fixed on several days after stimulation and stained with CD19, CD27 and CD138 antibodies. The upper graph in the diagram represents the fractions of $CD27^{high}$ expressing cells in the proliferated $CD19^+$ cells in dependence of days of stimulation. The lower graph shows the same for the additional expression of CD138.

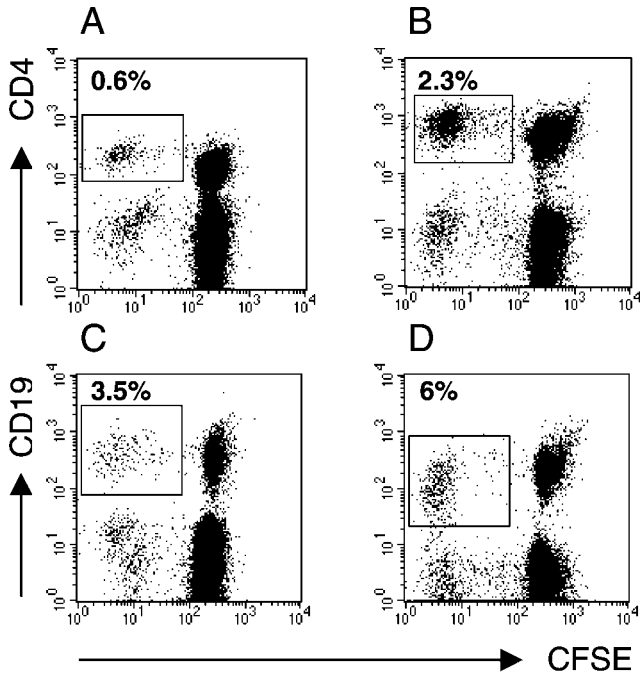

Figure 6. Nucleosome-reactive lymphocytes in SLE-patients and healthy donors: CFSE-labeled PBMC obtained from a healthy donor and an SLE-patient were stimulated with nucleosomes (20 μg/ml) for six days and stained with CD4 and CD19 antibodies. *A/C*: nucleosome-reactive proliferated $CD4^+$ Th cells (*A*) and $CD19^+$ B cells (*C*) in PBMC from an SLE-patient. *B/D*: nucleosome-reactive proliferated $CD4^+$ Th cells (*B*) and $CD19^+$ B cells (*D*) in PBMC from a healthy donor. Percentages refer to total numbers of $CD4^+$ Th cells or $CD19^+$ B cells, respectively.

Discussion

We have here performed the cytometric analysis of reactive cellular proliferation after *in vitro* antigenic stimulation based on labeling of peripheral mononuclear cells with carboxyfluorescein-diacetate succinimidyl ester (CFSE). During labeling of cells CFSE binds at random to secondary amino-groups of cellular proteins. This provides a uniform fluorescent label to all cells prior to the antigenic challenge. Cells reacting to the antigen by proliferation will lose 50% of their fluorescent label upon each cell division. This allows not only identification and enumeration of antigen-reactive cells, but also supplies information on the proliferative history of each of the cells. Furthermore the cytometric analysis of reactive lymphocyte proliferation in response to specific antigens as performed here has the advantage that the detected proliferation can be directly assigned to distinct lymphocyte subsets.

Firstly, we evaluated the possibility of a simultaneous cytometric detection of both, antigen-reactive proliferating T and B cells in one sample. By reducing the concentration of the CFSE staining solution during the labeling procedure it was possible to per-

form four colour analysis on the stimulated cells, allowing to assign proliferation directly to either $CD4^+$ Th cells, $CD8^+$ cytotoxic T cells or $CD19^+$ B cells in just one analysed sample. Interestingly, we observed that apart from clearly detectable Th cell proliferation also considerable high frequencies of B cells proliferated in response to the antigenic provocation *in vitro*. The sensitivity of a CFSE-based proliferation assay compared to the classical analysis of reactive proliferation based on the incorporation of tritiated thymidin has been already demonstrated (7). However, we show here that in addition this technology enables a simultaneous analysis of different types of antigen-reactive lymphocytes in one sample of cultivated cells.

Another advantage of the assay as it was performed here, is the possibility to further analyse antigen-reactive proliferating cells functionally, since CFSE-labeling does not alter cell viability nor cellular function (6). Antigen-reactive T cells can be characterized further as we demonstrate here by analysing cytokine secretion patterns of antigen-reactive proliferating Th cells after polyclonal restimulation *in vitro*. We show here that CFSE-labeled $CD4^+$ Th cells can be isolated from the antigen-stimulated cultures by magnetic separation and subsequently restimulated polyclonally using PMA and ionomycin. This enables a multiparameter intracellular cytokine analysis assigning cytokine production either to antigen-reactive $CFSE^{low}$ Th cells or $CFSE^{high}$ non-reactive resting Th cells. Using this experimental strategy we were able to detect allergen-reactive proliferating Th cells in both atopic and healthy donors. However, while overall frequencies of allergen-reactive proliferated Th cells did not differ drastically, we could detect obvious differences in the cytokine secretion patterns of proliferated Th cells. In allergen-reactive Th cells from the atopic donor we detected higher frequencies of exclusively IL-4 and IL-13 producing Th cells and lower frequencies of IFN-γ and IFN-γ/IL-4 coexpressing Th cells as compared to the allergen-reactive Th cells from the healthy donor. This result demonstrates that the mere quantification of antigen-reactive Th cell proliferation might be not sufficient to identify Th cells involved in pathological situations. A rather qualitative examination of Th cell responses is necessary to perform an accurate analysis.

In additional experiments we evaluated the phenotype of antigen-reactive proliferating $CD19^+$ B cells, which were detected after stimulation with the recall antigen tetanus toxoid. Firstly, we could show that after 6 days of *in vitro* stimulation a high percentage of proliferated $CD19^+$ B cells strongly expressed the CD27 molecule and in parallel downregulated the expression of CD20. This $CD27^{high}$ $CD20^{low}$ phenotype is characteristic in plasma blasts (9). Interestingly, antigen-reactive B cells needed at least 3–4 cell divisions to acquire the $CD27^{high}$ $CD20^{low}$ plasmablast phenotype. We could further show that a few of the antigen-reactive *in vitro* differentiated $CD27^{high}$ $CD20^{low}$ plasmablasts gained CD138 expression after 6 days of *in vitro* antigen stimulation. By this, we demonstrate that the here described method enables also a detailed analysis of antigen-reactive B cell proliferation and differentiation. Antigen-reactive B cells can be isolated directly at different stages of differentiation and analysed further either functionally in growth studies including the determination of differentiation factor requirements or molecularly for their V-genes' diversification.

Finally we adapted the technique for the simultaneous evaluation of autoantigen-reactive Th and B cell proliferation in patients suffering from systemic lupus erythematosus (SLE) and healthy control persons. Just by analysing the nucleosome-specific Th and B cell responses of a few SLE patients and healthy controls we obtained drastic

results. Surprisingly, both antigen-reactive Th and B cells were detected in both SLE patients and healthy donors. Whereas Th cell proliferation was detected frequently in 5 out of 13 SLE patients but also in 5 out of 10 healthy donors, B cell proliferation could only be detected in 3 SLE patients and 2 healthy controls. Although larger numbers of analyzed patients and normal healthy controls would be necessary to draw strong conclusions, our preliminary data is in line with other reports that autoantigen-reactive T cells specific for SLE-associated autoantigens can be detected also in healthy people (13). However, further tests are necessary, to prove the antigen-specificities of both proliferating nucleosome-reactive Th and B cells in our assays. Of note is that the same preparation of nucleosomes has been used for the analysis of nucleosomes-specific autoantibodies. No correlation was observed for the detection of nucleosome-reactive Th cell or B cell proliferation in SLE patients and anti-nucleosomal autoantibodies in the sera of the corresponding patients. None of the tested sera from the healthy donors was positive for anti-nucleosomal autoantibodies.

To summarize: We have demonstrated here the feasibility of a cytometric analysis of (auto)antigen-reactive cell proliferation based on CFSE-labeling of PBMC and subsequent antigenic *in vitro* provocation. The methodology proved to be effective for a simultaneous evaluation of antigen-reactive proliferating T and B cells in one sample. Antigen-reactive proliferation can accurately be assigned to distinct cell types and in addition antigen-reactive cells can be analyzed further phenotypically or functionally. Finally, by using this simple assay we have started to analyze autoantigen-reactive Th and B cell proliferation in SLE patients and healthy donors. A technology identifying antigen-specific cells according to their reactive proliferation enables unique options to analyze antigen-specific Th and B lymphocytes directly *ex vivo* and to isolate them for molecular and functional studies. This opens new perspectives to develop diagnostic and therapeutic strategies based on the identification and isolation of antigen-specific cells.

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